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Function of OPG as a Traffic Regulator for RANKL Is Crucial for Controlled Osteoclastogenesis

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ABSTRACT

The amount of the receptor activator of NF-κB ligand (RANKL) on the osteoblastic cell surface is considered to determine the magnitude of the signal input to osteoclast precursors and the degree of osteoclastogenesis. Previously, we have shown that RANKL is localized predominantly in lysosomal organelles, but little is found on the osteoblastic cell surface, and consequently, the regulated subcellular trafficking of RANKL in osteoblastic cells is important for controlled osteoclastogenesis. Here we have examined the involvement of osteoprotegerin (OPG), which is currently recognized as a decoy receptor for RANKL, in the regulation of RANKL behavior. It was suggested that OPG already makes a complex with RANKL in the Golgi apparatus and that the complex formation is necessary for RANKL sorting to the secretory lysosomes. It was also shown that each structural domain of OPG is indispensable for exerting OPG function as a traffic regulator. In particular, the latter domains of OPG, whose physiologic functions have been unclear, were indicated to sort RANKL molecules to lysosomes from the Golgi apparatus. In addition, the overexpression of RANK-OPG chimeric protein, which retained OPG function as a decoy receptor but lost the function as a traffic regulator, inhibited endogenous OPG function as a traffic regulator selectively in osteoblastic cells and resulted in the upregulation of osteoclastogenic ability despite the increased number of decoy receptor molecules. Conclusively, OPG function as a traffic regulator for RANKL is crucial for regulating osteoclastogenesis at least as well as that as a decoy receptor. © 2010 American Society for Bone and Mineral Research.

KEY WORDS: GOLGI APPARATUS; OPG; OSTEOCLASTOGENESIS; PROTEIN SORTING; RANKL

Introduction

Bone remodeling allows for adaptation to mechanical constraints and maintains homeostasis of phosphorus and calcium through coordinated phases composed of bone formation by osteoblasts and bone resorption by osteoclasts. The equilibrium between osteoblast and osteoclast activities is tightly regulated by many factors such as hormones, growth factors, and cytokines.[1,2] Excess activation of osteoclasts disturbs this equilibrium and culminates in bone destruction,[3] which is a common mechanism for the development of osteopenic disorders, including postmenopausal osteoporosis, rheumatoid arthritis, and lytic bone metastases.[4,5] Numerous studies have shown that the receptor activator of NF-κB (RANK) signaling pathway is crucial for the differentiation and activation of osteoclasts.[6–8] Therefore, the amount of RANK ligand (RANKL) on the osteoblastic cell surface, where RANKL binds to RANK through cell-to-cell contact and triggers downstream signaling in osteoclast precursors, is considered to determine the magnitude of the signal input and the degree of osteoclastogenesis.[9,10]

Previously, we have shown that most of the newly synthesized RANKL is transferred from the Golgi apparatus to the lysosomal storage compartment via the route involving vacuolar protein sorting 33a (Vps33a) in osteoblastic cells.[11] There also exists the minor pathway transporting RANKL from the Golgi apparatus to the plasma membrane.[11] In addition, we have shown that the stimulation of osteoblastic cells with RANK extracellular domain conjugated beads, which mimics contact with osteoclasts, induces translocation of RANKL from the lysosomal storage to the bead interface.[11] Recruitment of RANKL to the bead interface requires only binding of the RANK extracellular domain to the small amount of RANKL already localized at the cell surface, which might be supplied via the minor pathway. We also

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have shown that the suppression of Vps33a disrupted this regulated behavior of RANKL, resulting in the accumulation of RANKL in the Golgi apparatus and thus in upregulation of the cell surface RANKL leaked via the minor pathway in osteoblastic cells.[11] These findings indicate that regulation of RANKL at the protein trafficking level has an impact on osteoclastogenesis physiologically, and Vps33a-mediated transport of RANKL plays a key role.

However, there still remains the problem that RANKL molecules are transferred mostly to the plasma membrane when expressed in nonosteoblastic cells, such as HeLa cells and HEK293 cells,[11] whereas Vps33a is expressed ubiquitously in these cell lines.[12] In order to explain this discrepancy, it should be assumed that there are additional machineries that are required for RANKL transport from the Golgi apparatus to the secretory lysosomes in osteoblastic cells. Here we have focused on osteoprotegerin (OPG), which is expressed in osteoblastic cells as well as RANKL,[13,14] and we have investigated the involvement of OPG in RANKL subcellular trafficking. OPG is a homodimeric secretory protein consisting of N-terminal cysteine-rich domains (CRDs), following death domain homologous domains (DDHDs), and the C-terminal heparin-binding domain (HBD).[15] In vitro analyses have indicated that OPG functions as a decoy receptor for RANKL, inhibiting the RANK-RANKL interaction and thus osteoclastogenesis by tightly binding to RANKL via its CRD.[16,17] In vivo analyses using OPG-deficient mice, which develop severe early-onset osteoporosis with increased osteoclast numbers, supported the preceding theory about the physiologic function of OPG CRDs.[18,19] However, the physiologic function of DDHDs and the HBD of OPG was still unclear.

In this study, we have analyzed the involvement of OPG in subcellular trafficking of RANKL and have found that coexpression of OPG with RANKL is required for proper transfer of RANKL from the Golgi apparatus to the secretory lysosomes. Using various OPG mutants, we demonstrated the need for the HBD of OPG for proper RANKL trafficking. Finally, we have examined the physiologic significance of OPG function as a traffic regulator for RANKL in osteoblastic cells.

Materials and Methods

Animals

All animal procedures were approved by the Institutional Animal Care and Use Committee of Graduate School of Medicine, University of Tokyo. C57/BL6 wild-type (WT) mice were purchased from Japan SLC (Shizuoka, Japan). *OPG*$^{-/-}$ mice were obtained from Japan Clea Co. (Tokyo, Japan).[18,19]

Cell culture

HeLa cells from the RIKEN Cell Bank (RCB) were cultured in DMEM containing 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA), nonessential amino acids (Invitrogen), and penicillin-streptomycin (PCSM; Invitrogen). ST2 cells from RCB were cultured in α-MEM containing 10% FBS and PCSM. 293FT cells from Invitrogen were cultured in DMEM containing 10% FBS, nonessential amino acids, PCSM, and G-418 (Nacalai Tesque, Kyoto, Japan).

Preparation of osteoblastic cells and bone marrow cells

Primary osteoblastic cells were isolated from the calvaria of 1- to 4-day-old newborn male C57/BL6 mice and cultured in type I collagen gel (cell matrix type IA; Nitta Gelatin, Inc., Osaka, Japan) using a previously described method with some modifications.[20] Bone marrow cells obtained from the tibias of 6- to 8-week-old male C57/BL6 mice were cultured in α-MEM containing 10% FBS in the presence of macrophage colony-stimulating factor (M-CSF; 5 ng/mL; Cosmo Bio, Tokyo, Japan) using a previously described method with some modifications.[21] Nonadherent cells were harvested and used in the coculture assay with ST2 or primary osteoblastic cells.

Construction and transfection of expression vectors

Mouse OPG, RANK, lysosome-associated membrane protein 1 (LAMP1), calnexin, and 58K Golgi protein were cloned from cDNA of ST2 cells. The immunoglobulin Fc region and asialoglycoprotein receptor 1 (Asgr1) were cloned from the cDNA of hematocytes and liver from C57/BL6 mice, respectively. Each OPG mutant, as shown in Fig. 2*A*, was generated by overlap extension PCR and/or site-directed mutagenesis using QuickChange (Stratagene, La Jolla, CA, USA). Wild-type OPG and mutants were subcloned into pcDNA3.1 (Invitrogen). LAMP1, calnexin, and 58K Golgi protein were subcloned into pmKO1-MN1 (MBL, Aichi, Japan) (LAMP1-KuOr, calnexin-KuOr, and G58K-KuOr, respectively) and wild-type OPG into pEGFP-N1 (Clontech, Palo Alto, CA, USA) (OPG-GFP) and Asgr1 into pEGFP-C1 (Clontech) (GFP-Asgr1). Previously cloned mouse RANKL and Vps33a[11] were subcloned into pEGFP-C1 (Clontech) (GFP-RANKL), pDsRed-monomer-Hyg-C1 (Clontech) (DsRed-RANKL), and pcDNA3.1 (Invitrogen) after fusing the His-tag at the C terminus (H-Vps33a). Each construct was introduced in HeLa, ST2, and primary osteoblastic cells using FuGENE 6 (Roche Diagnostics, Indianapolis, IN, USA) or introduced into 293FT cells using Lipofectamine 2000 Reagent (Invitrogen).

Recombinant OPG protein purification

OPG-H and RANK-CRD-OPG-ΔCRD recombinant proteins were produced by transfecting to 293FT cells. Their whole media were applied to Profinity IMAC Ni-Chaged Resin (Bio-Rad, Hercules, CA, USA), washed with PBS containing 20 mM imidazole, and then eluted with PBS containing 100 mM imidazole. The buffer was then changed to imidazole-free PBS, and the protein concentration was quantified using the bicinchoninic acid (BCA) protein assay kit (Thermo Scientific, Bremen, Germany) according to the manufacturer's protocol.

Immunostaining and observation of live cells

Primary cultured mice osteoblastic cells were cultured in α-MEM containing 10% FBS and PCSM with 10 nM 1α,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$; Sigma, St. Louis, MO, USA] and 100 nM dexamethasone (Dex; Sigma) for 72 hours before fixation. Cells were fixed with formaldehyde, permeabilized with Triton X-100, and then subjected to immunostaining using goat anti-RANKL antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-LAMP1 antibody (Sigma), or mouse anti-58K Golgi protein

antibody (Abcam, Cambridge, UK) as the primary antibody, and the corresponding secondary antibodies were labeled with Alexa Fluor dye (Invitrogen) diluted in Can Get Signal immunostain (Toyobo, Osaka, Japan). Fluorescence was detected using a confocal laser-scanning microscope (Fluoview FV1000, Olympus, Melville, NY, USA) equipped with a UPLSAPO ×100 objective lens (numerical aperture 1.40; Olympus).

Immunoprecipitation assay

To analyze the interaction between RANKL and each of the OPG mutants, both GFP-RANKL and each of the OPG mutants were transiently introduced in HeLa cells. To analyze the interaction between RANKL and Vps33a, GFP-RANKL and H-Vps33a, either with or without nontagged OPG, were transiently introduced in HeLa cells. The cells were collected and lysed in lysis buffer (PBS containing 0.1% NP40, 1 mg/mL of PMSF, and a protease inhibitor cocktail; Roche). The lysate was immunoprecipitated using a mouse anti-His-tag antibody (Sigma). The precipitates and cell lysates were subjected to immunoblotting with rabbit anti-GFP antibody (Invitrogen) or mouse anti-His-tag antibody as the primary antibody and horseradish peroxidase (HRP)–labeled donkey anti-rabbit IgG antibody (GE Healthcare, Buckinghamshire, UK) or HRP-labeled sheep anti-mouse IgG antibody (GE Healthcare) as the secondary antibody. The blots were detected using the ECL plus detection reagent (GE Healthcare).

Biotinylation assay

To detect GFP-RANKL expression on the cell surface in primary osteoblastic or ST2 cells, cell surface proteins were biotinylated using EZ-Link sulfo-NHS-SS-biotin (Thermo Scientific) following the manufacturer's protocol, and cells were harvested and lysed in lysis buffer. Biotinylated proteins were collected using MagnaBind streptavidin magnetic beads (Pierce) following the manufacturer's protocol and subjected to immunoblotting. The blots were probed, and proteins were detected with rabbit anti-GFP antibody (Invitrogen) or goat anti-Na^+,K^+-ATPase antibody (Santa Cruz) as the primary antibody and HRP-labeled donkey anti-rabbit IgG antibody (GE Healthcare) or HRP-labeled donkey anti-goat IgG antibody (Santa Cruz) as the corresponding secondary antibody.

Examination for lysosomal and proteasomal degradation

ST2 cells expressing GFP-RANKL and OPG-H were treated with 100 μM chloroquine (Nacalai Tesque) or 10 μM MG132 (Nacalai Tesque) for 5 hours. Cells were collected and lysed in lysis buffer. Cell lysates were subjected to mouse anti-His-tag antibody (Sigma), rabbit anti-GFP antibody (Invitrogen), and rabbit anti-α-tubulin antibody (Abcam) as the primary antibody and the corresponding secondary antibody.

ELISA

HeLa cells, mouse primary osteoblastic cells, or ST2 cells were seeded in 48-well plates. If needed, cells were treated with 100 μM chloroquine (Nacalai Tesque) for 5 hours and 10 nM $1,25(OH)_2D_3$ and 100 nM Dex for 72 hours. After recovery of the culture medium, cells were lysed in lysis buffer, and the concentrations of RANKL and/or OPG in the culture medium and cell lysates were determined using Quantikine immunoassay kits (R&D Systems, Minneapolis, MN, USA) following the manufacturers' protocols.

Coculture assay

Coculture was performed following a previous report.[22] For measuring tartrate-resistant acid phosphatase (TRACP) enzymatic activity, bone marrow cells were cocultured with ST2 or primary osteoblastic cells (transiently expressing OPG mutants where necessary) for 7 days in a 96-well plate containing 10 nM $1,25(OH)_2D_3$ and 100 nM Dex, either with or without OPG recombinant protein (100 ng/mL). The culture medium was replenished every 3 days with fresh medium containing the OPG recombinant protein (100 ng/mL) if needed. TRACP enzymatic activity, which was evaluated by measuring *p*-nitrophenol production from *p*-nitrophenyl phosphate under tartaric acidic conditions, was determined using a TRACP assay kit (TaKaRa, Shiga, Japan) following the manufacturer's protocol.

mRNA quantification

The amount of mRNA was quantified by reverse transcription and real-time quantitative PCR with SYBR GreenER qPCR SuperMix Universal (Invitrogen), Chromo4 (Bio-Rad), and the associated software using the following primers: 5′-CGC TTT CAA AGA GTG CTA CC-3′ and 5′-GTT GAG CTG AAG CTT CTT GG-3′ for mouse Vps33a, 5′-ACC CAG AAA CTG GTC ATC AGC-3′ and 5′-CTG CAA TAC ACA CAC TCA TCA CT-3′ for mouse OPG, 5′-GTC TGT AGG TAC GCT TCC CG-3′ and 5′-CAT TTG CAC ACC TCA CCA TCA AT-3′ for mouse RANKL, and 5′-TTC AAC ACC CCA GCC ATG TAG G-3′ and 5′-GTG GTG GTG AAG CTG TAG CC -3′ for mouse β-actin.

Statistical analysis

All data are expressed as mean ± SD from three or four independent determinations. Statistical analysis was performed using Student's *t* test or an analysis of variance (ANOVA) followed by the Student-Newman-Keuls test where applicable.

Results

RANKL localization is affected by coexpression of OPG in nonosteoblastic cells

First, we analyzed the involvement of OPG in subcellular trafficking of RANKL using nonosteoblastic HeLa cells, in which RANKL or OPG is not expressed endogenously, by introducing RANKL fused with GFP at the N terminal (GFP-RANKL) with or without OPG. Without coexpression of OPG, GFP-RANKL is expressed predominantly on the plasma membrane (Fig. 1*A*), whereas GFP-RANKL is observed largely in lysosomes when coexpressed with wild-type OPG fused with His-tag at the C terminal (OPG-H) (Fig. 1*B*). This observation is comparable with that seen when GFP-RANKL is introduced in osteoblastic ST2 cells, in which RANKL and OPG are expressed endogenously (Fig. 1*C*). As a lysosomal marker in live cell staining, we introduced LAMP1 fused with Kusabira-Orange at the C terminal (LAMP1-KuOr) simultaneously.[23] On the other hand, addition of recombinant OPG-H protein (100 ng/mL) to the culture medium

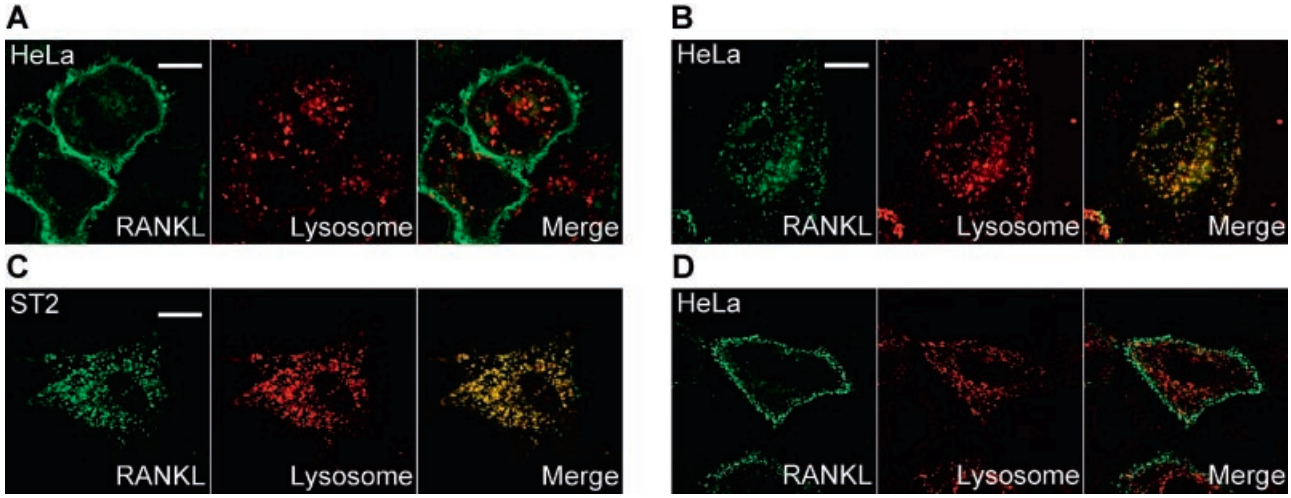

Fig. 1. RANKL localization is affected by coexpression of OPG in nonosteoblastic cells. (*A*) GFP-RANKL was introduced with LAMP1-KuOr in nonosteoblastic HeLa cells. GFP-RANKL was expressed predominantly on the plasma membrane. LAMP1-KuOr was introduced as a lysosomal marker. (*B*) GFP-RANKL was introduced with OPG-H and LAMP1-KuOr in HeLa cells. GFP-RANKL was observed largely in the lysosomes. (*C*) GFP-RANKL was introduced with LAMP1-KuOr in osteoblastic ST2 cells. This observation was comparable with panel *B*. (*D*) GFP-RANKL was introduced with LAMP1-KuOr in HeLa cells, and recombinant OPG-H protein (100 ng/mL) was added simultaneously. GFP-RANKL was expressed predominantly on the plasma membrane. Each bar indicates 10 μm.

did not change RANKL localization on the plasma membrane to lysosomes (Fig. 1*D*). A previous study reported the rapid disappearance of OPG from the culture medium of 293RL cells,[24] so in order to check this we measured the amount of OPG protein remaining in the culture medium by ELISA. At the time of observation (24 hours after addition to the medium), increased levels of OPG protein were detected in the medium (19.3 ± 1.5 ng/mL) compared with those detected in the case of coexpression (5.2 ± 1.1 ng/mL). These observations indicate that RANKL localization in lysosomes when coexpressed with OPG is not due to internalization from the plasma membrane stimulated by binding to the secreted OPG.

OPG structural integrity is necessary for regulating RANKL localization

Next, we investigated the contribution of each OPG structural domain in RANKL trafficking. In order to examine this point, we constructed various OPG mutants (Fig. 2*A*). We analyzed the interaction of each His-tagged OPG mutant with RANKL under conditions of coexpression by immunoprecipitation. Strong interactions between each OPG mutant and RANKL were observed, except for the CRD-deletion mutant (OPG-ΔCRD; Fig. 2*B*). The results are not unexpected, considering that OPG binds tightly to RANKL through its CRD domains.[16,17] We also investigated the subcellular localization of RANKL in HeLa cells under coexpression conditions (Fig. 3). Localization of GFP-RANKL was not affected by coexpression of either the monomeric protein construct of only CRD (OPG-CRD; Fig. 3*A*) or the dimeric fusion protein construct of OPG CRD and the immunoglobulin Fc region (OPG-CRD-Fc; Fig. 3*B*). These results suggest that the latter half domains of OPG are necessary for regulating RANKL trafficking. When the deletion mutant of HBD (OPG-ΔHBD) was coexpressed, GFP-RANKL was not expressed on the plasma membrane and tended to accumulate mainly in the Golgi apparatus and partially in the endoplasmic reticulum (Fig. 3*C*). In order to confirm the importance of HBD, we prepared a construct with mutations in all lysine and arginine residues in HBD to alanine (OPG-KR-A) because HBD exerts its function of interacting with proteoglycans via its cationic amino acids.[25] GFP-RANKL localization when coexpressed with OPG-KR-A showed the pattern of accumulation mainly in the Golgi apparatus and partially in the endoplasmic reticulum, which is comparable with that observed in the case of OPG-ΔHBD (Fig. 3*D*). These observations were confirmed by coexpressing calnexin fused with Kusabira-Orange at the C terminus (calnexin-KuOr), which is a marker for the endoplasmic reticulum,[26] and 58K Golgi protein fused with Kusabira-Orange at the C terminus (G58K-KuOr), which is a marker for the Golgi apparatus.[27] These results indicate that HBD plays an important role in RANKL export from the Golgi apparatus and also indicate that RANKL and OPG interact already in the endoplasmic reticulum and/or in the Golgi apparatus. To confirm the latter point, we analyzed the effect of OPG fused with Lys-Asp-Glu-Leu (KDEL) peptide at the C terminus (OPG-KDEL). Many resident soluble proteins in the endoplasmic reticulum have a KDEL sequence as a retention signal that is recognized by KDEL receptors in the *cis*-Golgi network and thus are transported retrogradely from the Golgi apparatus to the endoplasmic reticulum.[28] When GFP-RANKL and OPG-KDEL were coexpressed in HeLa cells, GFP-RANKL accumulated mainly in the endoplasmic reticulum (Fig. 3*E*).

Finally, we checked the need of CRD for regulating RANKL trafficking because CRD is considered to be necessary for interacting with RANKL. Coexpression of OPG-ΔCRD did not affect GFP-RANKL localization on the plasma membrane in HeLa cells, as we expected (Fig. 3*F*). However, RANKL trafficking was also not affected by coexpression of the chimeric protein construct that has RANK CRD instead of OPG CRD (RANK-CRD-OPG-ΔCRD; Fig. 3*G*). This finding suggests that the function of OPG CRD is not restricted to binding with RANKL and that

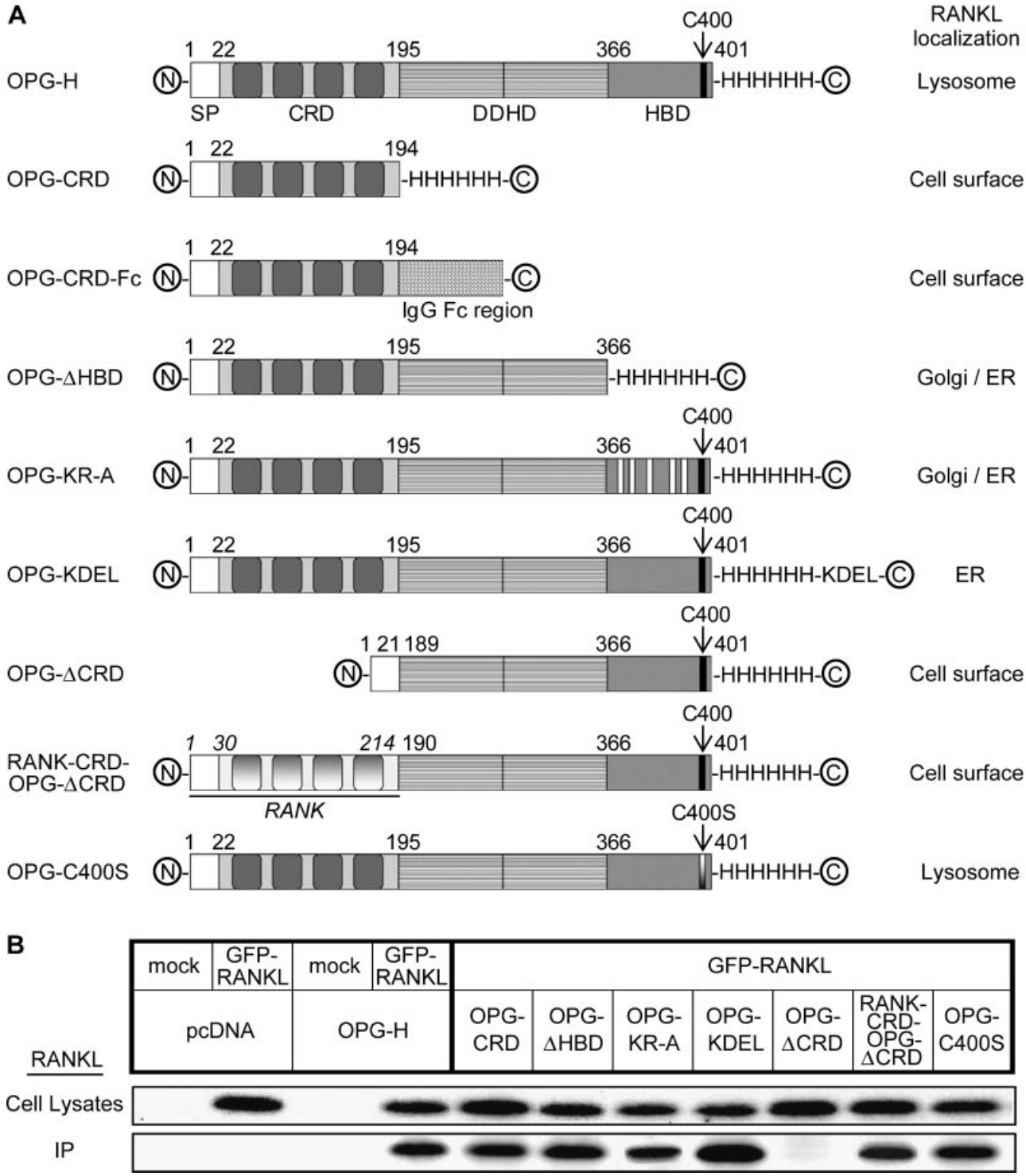

Fig. 2. Gene constructs for OPG mutants. (*A*) The abbreviations used are as follows: SP = signal peptide; CRD = cysteine-rich domain; DDHD = death domain homologous domain; HBD = heparin-binding domain. (*B*) The HeLa cells transfected with GFP-RANKL and each His-tagged OPG mutant were lysed and immunoprecipitated (IP) with an anti-His-tag antibody. Except for the OPG-ΔCRD mutant, an interaction between RANKL and each of the OPG mutants was detected.

OPG structural integrity is necessary for regulating RANKL trafficking. In addition, we investigated the need for covalent dimerization via the disulfide bond at the cysteine residue adjacent to the C terminus (C400). GFP-RANKL showed a clear lysosomal localization pattern when coexpressed with a point-mutant construct of C400 to serine (OPG-C400S; Fig. 3H). A previous report showed that DDHDs and HBD of OPG contribute to the noncovalent dimerization of OPG,[29] and thus the structural integrity was not considered to be affected when C400 was substituted with a serine residue. The results of the experiments using the various OPG mutants are summarized in Fig. 2*A*, adjacent to the diagram of each construct.

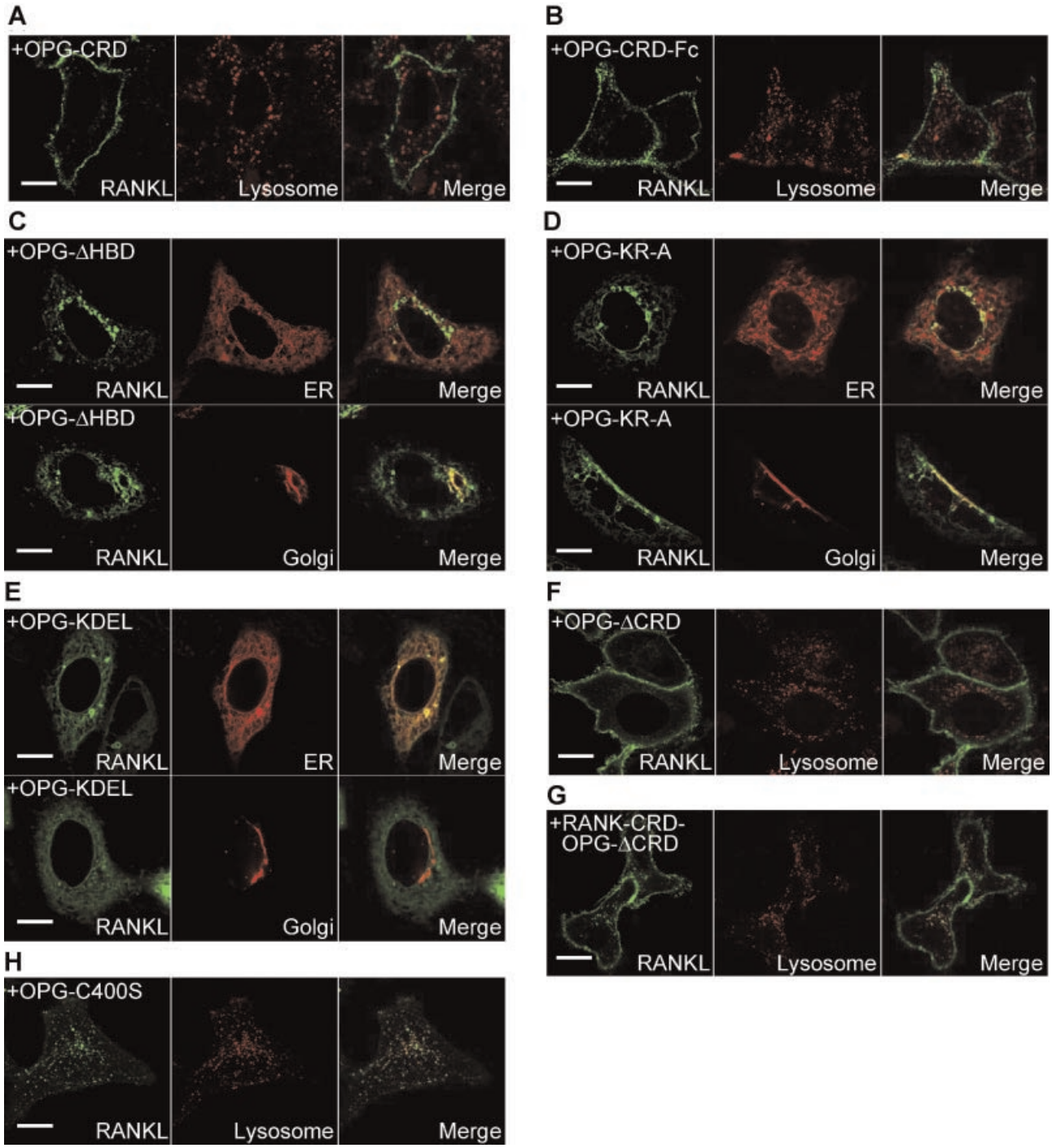

Fig. 3. OPG structural integrity is necessary for regulating RANKL localization. (*A*, *B*) GFP-RANKL and OPG-CRD (monomeric) or OPG-CRD-Fc (dimeric) were introduced with LAMP1-KuOr in HeLa cells. GFP-RANKL was expressed predominantly on the plasma membrane. (*C*, *D*) GFP-RANKL and OPG-ΔHBD or OPG-KR-A were introduced with calnexin-KuOr or G58K-KuOr in HeLa cells. GFP-RANKL tended to accumulate mainly in the Golgi apparatus but also showed some accumulation in the endoplasmic reticulum. Calnexin-KuOr was introduced as an endoplasmic reticulum marker (*upper panels*) and G58K-KuOr as a Golgi marker (*lower panels*). (*E*) GFP-RANKL and OPG-KDEL were introduced into HeLa cells along with calnexin-KuOr (*upper panels*) or G58K-KuOr (*lower panels*). GFP-RANKL tended to accumulate mainly in the endoplasmic reticulum. (*F*, *G*) GFP-RANKL and OPG-ΔCRD or RANK-CRD-OPG-ΔCRD were introduced with LAMP1-KuOr in HeLa cells. GFP-RANKL was expressed predominantly on the plasma membrane. (*H*) GFP-RANKL and OPG-C400S were introduced with LAMP1-KuOr in HeLa cells. GFP-RANKL showed a lysosomal localization pattern. Each bar indicates 10 μm.

RANKL is accumulated in the Golgi apparatus in *OPG*$^{-/-}$ osteoblastic cells

In order to clarify the effect of OPG on RANKL subcellular trafficking in osteoblastic cells, we investigated RANKL localization in mouse primary osteoblastic cells. Immunostaining using anti-RANKL antibody showed that endogenous RANKL was localized predominantly in lysosomes in *OPG*$^{+/+}$ osteoblastic cells (Fig. 4*A*). As a lysosomal marker, LAMP1 was costained by anti-LAMP1 antibody. On the other hand, RANKL tended to

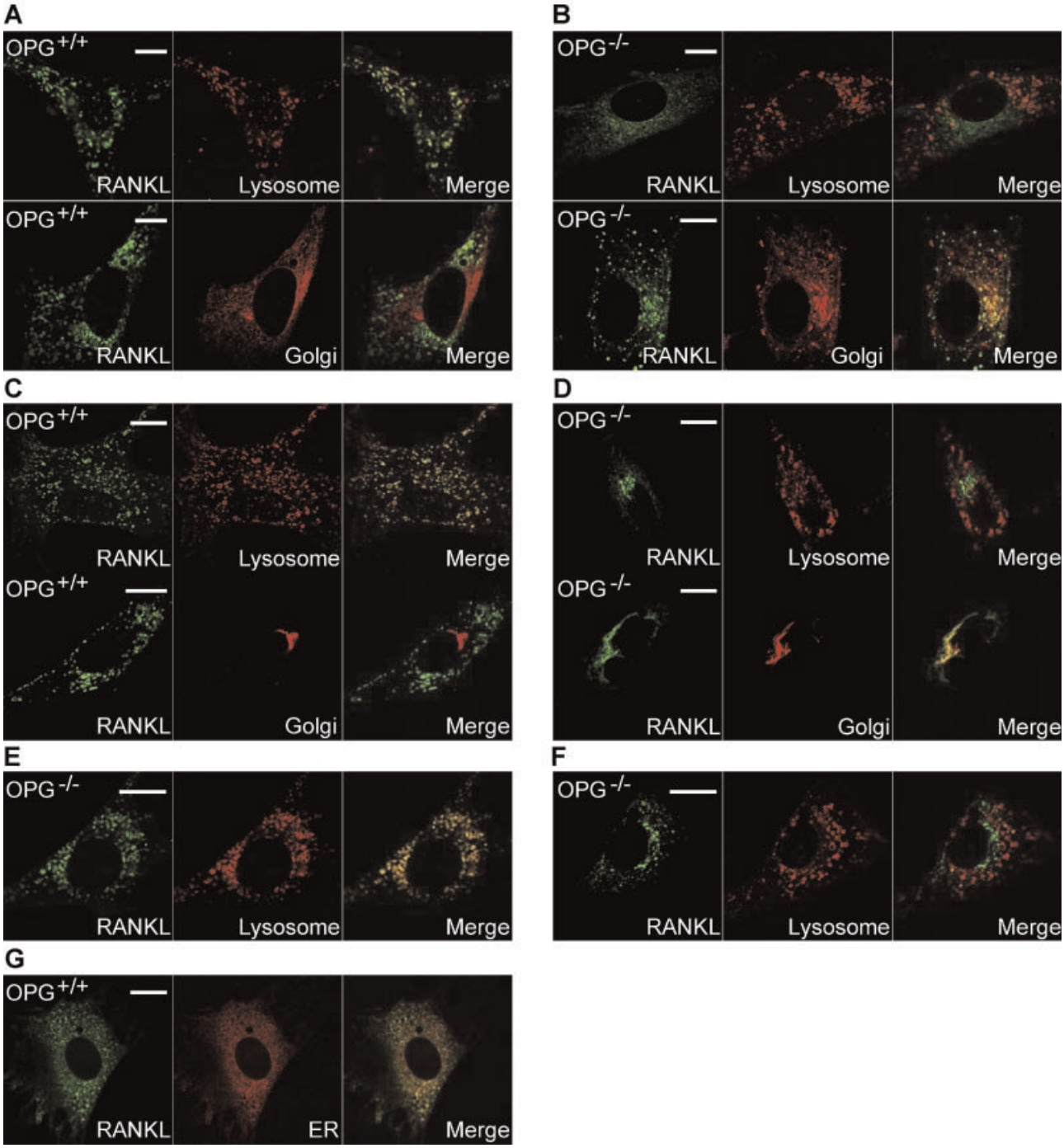

Fig. 4. RANKL accumulated in the Golgi apparatus in $OPG^{-/-}$ osteoblastic cells. (*A*) Immunostaining using anti-RANKL antibody showed that endogenous RANKL was localized predominantly in the lysosomes in $OPG^{+/+}$ osteoblastic cells. As a lysosomal marker, LAMP1 was costained with anti-LAMP1 antibody. Costaining with 58K Golgi protein, which is a marker for the Golgi apparatus, also was performed using anti-58K Golgi protein antibody. (*B*) In $OPG^{-/-}$ osteoblastic cells, immunostaining using anti-RANKL antibody showed that endogenous RANKL tended to accumulate in the Golgi apparatus and was not colocalized with LAMP1. (*C*, *D*) GFP-RANKL was expressed transiently in primary osteoblastic cells, and LAMP1-KuOr (*upper panels*) or G58K-KuOr (*lower panels*) was coexpressed. These observations were comparable with those in immunostaining of endogenous RANKL (*A*, *B*). (*E*) When GFP-RANKL was introduced with OPG-H in $OPG^{-/-}$ osteoblastic cells, a lysosomal localization pattern of RANKL was recovered. (*F*) The addition of OPG recombinant protein (100 ng/mL) in the culture medium along with introduction of GFP-RANKL and LAMP1-KuOr (*upper panels*), or G58K-KuOr (*lower panels*) into $OPG^{-/-}$ osteoblastic cells did not affect the localization pattern of GFP-RANKL in the Golgi apparatus. (*G*) Introducing OPG-KDEL with GFP-RANKL in $OPG^{+/+}$ osteoblastic cells resulted in the accumulation of GFP-RANKL in the endoplasmic reticulum. Calnexin-KuOr was cointroduced as an endoplasmic reticulum marker. Each bar indicates 10 μm.

accumulate in the Golgi apparatus (confirmed by costaining with anti-58K Golgi protein antibody) and was not colocalized with LAMP1 in *OPG*$^{-/-}$ primary osteoblastic cells (Fig. 4*B*). These observations were confirmed by introducing GFP-RANKL with LAMP1-KuOr or G58K-KuOr in primary osteoblastic cells (Fig. 4*C*, *D*).

Next, we performed rescue experiments using *OPG*$^{-/-}$ osteoblastic cells. Lysosomal localization of RANKL was recovered when GFP-RANKL was introduced with OPG-H in *OPG*$^{-/-}$ osteoblastic cells (Fig. 4*E*). On the other hand, the simultaneous addition of OPG-H recombinant protein to the medium when introducing GFP-RANKL in *OPG*$^{-/-}$ osteoblastic cells did not affect the localization pattern of GFP-RANKL (Fig. 4*F*), although higher levels of OPG protein were detected in the culture medium (17.6 ± 2.7 ng/mL) compared with those under OPG coexpression conditions (3.5 ± 0.7 ng/mL). In addition, coexpression of OPG-KDEL in *OPG*$^{+/+}$ osteoblastic cells resulted in the accumulation of GFP-RANKL in the endoplasmic reticulum (Fig. 4*G*). These findings are comparable with the observations in the experiments using HeLa cells, suggesting that OPG interacts with RANKL in the endoplasmic reticulum or in the Golgi apparatus and also plays an important role in RANKL subcellular trafficking in osteoblastic cells.

RANKL accumulation in the Golgi apparatus results in an increase in RANKL transported to the cell surface via the minor pathway in *OPG*$^{-/-}$ osteoblastic cells

The observations in the preceding experiments are consistent with our previous report that RANKL accumulation in the Golgi apparatus was observed when the major export pathway of RANKL from the Golgi apparatus was disrupted by Vps33a suppression in osteoblastic cells.[11] However, there remains a question about these observations in osteoblastic cells because RANKL can be transported efficiently to the cell surface in nonosteoblastic HeLa cells (Fig. 1*A*). Therefore, in order to determine whether the transport pathway of membrane protein to the cell surface is suppressed nonselectively in osteoblastic cells, we investigated the localization of a reference protein. We selected Asgr1 as a reference protein because it is known to be a single-pass type II membrane protein as well as RANKL, and its subcellular trafficking in nonosteoblastic cells is well characterized.[30] Asgr1 fused with GFP at the N terminus (GFP-Asgr1) showed predominant localization on the plasma membrane (Fig. 5*A*), indicating that the transport pathway of RANKL to the cell surface is suppressed selectively in osteoblastic cells. We also have shown that RANKL accumulation in the Golgi apparatus in Vps33a-suppressed conditions resulted in the upregulation of RANKL transfer to the plasma membrane via the pathway that is considered to be minor under normal conditions.[11] Therefore, in order to determine the amount of RANKL on the cell surface under *OPG* knockout conditions, we performed a biotinylation assay. The amount of exogenously introduced GFP-RANKL on the cell surface in *OPG*$^{-/-}$ osteoblastic cells was much greater than that in *OPG*$^{+/+}$ osteoblastic cells (Fig. 5*B*). This result can explain the fact that *OPG*$^{-/-}$ mice exhibit an osteoporotic phenotype[18,19] because the large increase in the amount of RANKL localized on the cell surface is considered to result in the uncontrollable activation of osteoclasts. We also confirmed this observation by quantitation of endogenous RANKL in osteoblastic cells using ELISA. Under *OPG*$^{-/-}$ conditions, the amount of RANKL detected in the culture medium was greatly increased compared with that under *OPG*$^{+/+}$ conditions (Fig. 5*C*). These results indicate that the amount of endogenous RANKL localized at the cell surface is greatly increased under *OPG*$^{-/-}$ conditions.

Interaction between RANKL and Vps33a is greatly enhanced by coexpression of OPG

Previously, we identified Vps33a as a protein interacting with RANKL using a protein pull-down method, and we have shown that Vps33a is involved in the transfer of RANKL from the Golgi apparatus to the secretory lysosomes in osteoblastic cells.[11] In order to clarify the engagement between OPG and Vps33a, the interaction between RANKL and Vps33a was investigated under conditions of OPG coexpression. GFP-RANKL and Vps33a (fused with a His-tag at the N terminus; H-Vps33a) were introduced into HeLa cells either with or without nontagged OPG, followed by cell lysis and immunoprecipitation with an anti-His-tag antibody. We found that the interaction between RANKL and Vps33a was greatly enhanced under OPG coexpression conditions (Fig. 5*D*), whereas OPG expression did not affect endogenous *Vps33a* mRNA levels in primary osteoblastic cells (Fig. 5*E*) or the level of the introduced H-Vps33a protein in HeLa cells (Fig. 5*D*).

In addition, we investigated the post-Golgi behavior of OPG in osteoblastic cells using OPG fused with GFP at the C terminus (OPG-GFP) and RANKL fused with DsRed at the N terminus (DsRed-RANKL). As a result, OPG-GFP was observed mainly in lysosomes with DsRed-RANKL (Fig. 5*F*), suggesting that OPG was transported with RANKL to lysosomes in osteoblastic cells. Then, in order to determine whether OPG was degraded in lysosomes, we compared the OPG-H expression level with or without chloroquine treatment, which is known to inhibit lysosomal function.[24] An increase in the expression level of OPG-H was observed with chloroquine treatment (Fig. 5*G*), whereas the GFP-RANKL expression level appeared to be unchanged.[11] We also confirmed this observation by quantification of endogenous OPG in osteoblastic cells using ELISA (Fig. 5*H*).

Selective inhibition of endogenous OPG function as a traffic regulator for RANKL promotes the osteoclastogenic ability of osteoblastic cells

Finally, we examined the physiologic significance of OPG function as a traffic regulator for RANKL. Because OPG is also known to function as a decoy receptor for RANKL,[16,17] selective inhibition of OPG function as a traffic regulator is necessary. Considering the results of a series of experiments using HeLa cells, the RANK-CRD-OPG-ΔCRD construct was a candidate that would compete with endogenous OPG for binding to RANKL in the Golgi apparatus and selectively inhibit OPG function as a traffic regulator. In ST2 cells, GFP-RANKL was observed mainly in the Golgi apparatus when coexpressed with RANK-CRD-OPG-ΔCRD (Fig. 6*A*). This observation was confirmed in *OPG*$^{+/+}$ osteoblastic cells (Fig. 6*B*). It also was confirmed that RANK-CRD-OPG-ΔCRD did not rescue RANKL localization in *OPG*$^{-/-}$ osteoblastic cells (Fig. 6*C*). These observations indicated that

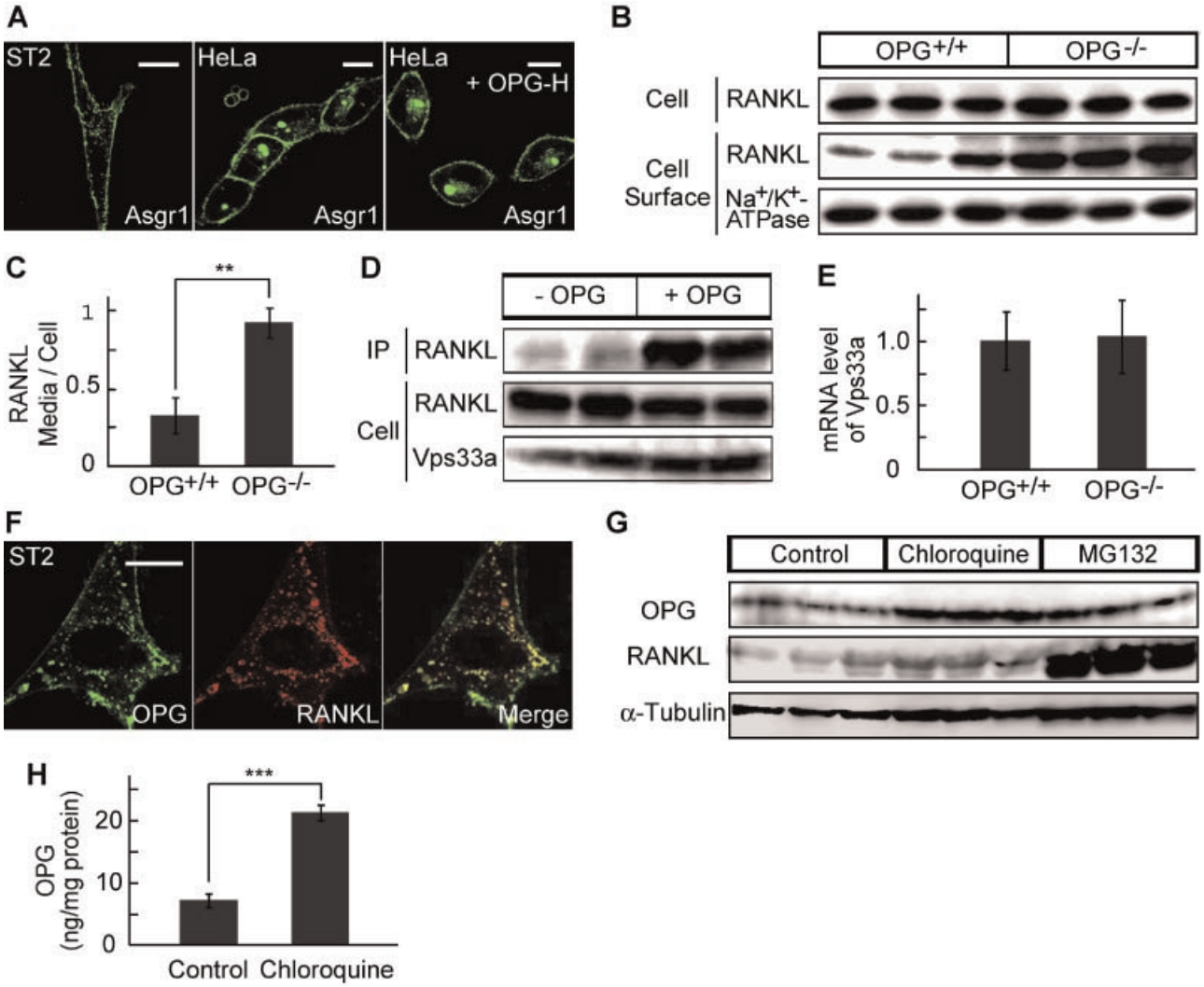

Fig. 5. RANKL accumulation in the Golgi apparatus results in an increase in RANKL transported to the cell surface via the minor pathway in $OPG^{-/-}$ osteoblastic cells. (*A*) GFP-Asgr1 was introduced into ST2 or HeLa cells with or without OPG-H. GFP-Asgr1 showed predominant localization on the plasma membrane in any cases. (*B*) Cell surface expression level of GFP-RANKL in osteoblastic cells was quantitated using a biotinylation assay. The cell lysates and cell surface proteins were subjected to immunoblotting and probed with anti-GFP antibody. The amount of exogenously introduced GFP-RANKL on the cell surface in $OPG^{-/-}$ osteoblastic cells was much greater than that in $OPG^{+/+}$ osteoblastic cells. Na^+,K^+-ATPase (used as a reference membrane protein) was probed with an anti-Na^+,K^+-ATPase antibody. (*C*) Endogenous RANKL concentration of culture medium and cell lysates in primary osteoblastic cells was quantitated using ELISA. Cells were treated with 10 nM $1,25(OH)_2D_3$ and 100 nM Dex for 72 hours. Under $OPG^{-/-}$ conditions, the ratio of the amount of RANKL detected in the culture medium to that in the cell lysates was markedly increased compared with that under $OPG^{+/+}$ conditions. Data are represented as mean ± SD. $^{**}p < .01$. (*D*) After GFP-RANKL and H-Vps33a were introduced into HeLa cells with or without nontagged OPG, cells were lysed and immunoprecipitated with an anti-His-tag antibody. The interaction between RANKL and Vps33a was greatly enhanced by OPG coexpression. (*E*) The mRNA levels of *Vps33a* in both $OPG^{+/+}$ and $OPG^{-/-}$ primary osteoblastic cells are similar. (*F*) OPG-GFP and DsRed-RANKL were introduced into ST2 cells. OPG-GFP was observed largely in lysosomes with DsRed-RANKL. Each bar indicates 10 μm. (*G*) ST2 cells expressing OPG-H and GFP-RANKL were either untreated or treated with 100 μM chloroquine (a lysosomal inhibitor) or 10 μM MG132 (a proteasomal inhibitor) for 5 hours. The cell lysates were subjected to immunoblotting and probed with anti-His-tag antibody or anti-GFP antibody. An increase in the expression level of OPG-H was observed with chloroquine treatment. α-Tubulin (probed with anti-α-tubulin antibody) was used as a positive control. (*H*) Endogenous OPG concentration of the cell lysates in $OPG^{+/+}$ osteoblastic cells was quantitated by ELISA. Cells were untreated or treated with 100 μM chloroquine for 5 hours. An increase in the expression level of OPG was observed with chloroquine treatment. Data are represented as mean ± SD. $^{***}p < .001$.

RANK-CRD-OPG-ΔCRD competes with OPG for RANKL binding in the Golgi apparatus and disrupts the regulated trafficking of RANKL when introduced into osteoblastic cells.

Next, we investigated the effect of RANK-CRD-OPG-ΔCRD overexpression on osteoclastogenic ability. The biotinylation assay showed that the cell surface GFP-RANKL was mainly increased with coexpression of RANK-CRD-OPG-ΔCRD in ST2 cells (Fig. 6*D*). In addition, the osteoclastogenic ability was examined as the tartrate-resistant acid phosphatase (TRACP) activity using a coculture assay with mouse bone marrow cells. As a result, the osteoclastogenic ability was significantly increased in ST2 cells transfected with the RANK-CRD-OPG-ΔCRD construct, whereas

that in OPG-H-transfected ST2 cells was slightly reduced (Fig. 6*E*). Contrary to this result, the osteoclastogenic ability was markedly inhibited when RANK-CRD-OPG-ΔCRD recombinant protein was added to the culture medium for the coculture assay system, showing that this recombinant protein can function as a decoy receptor for RANKL as well as OPG (Fig. 6*F*). Under these conditions, we checked that the mRNA levels of endogenous *OPG* or *RANKL* were not changed by the introduction of RANK-CRD-OPG-ΔCRD (Fig. 6*G*). Also, the molar ratio of RANKL/OPG at the protein level was measured by ELISA. While the molar ratio of

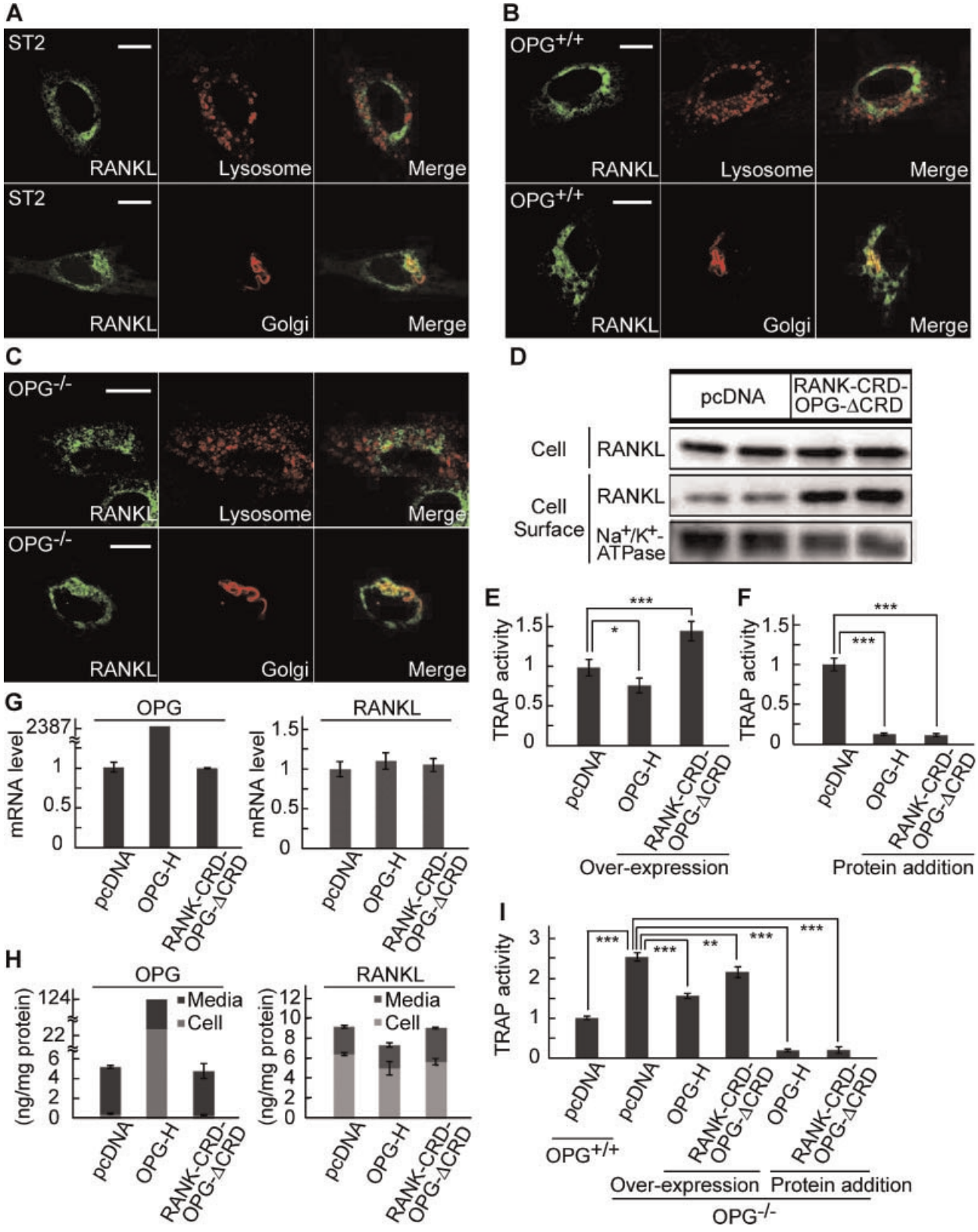

Fig. 6.

RANKL/OPG was decreased significantly in the case of OPG-H overexpression (0.21 ± 0.05), the introduction of RANK-CRD-OPG-ΔCRD did not affect the total expression levels of RANKL or OPG, which resulted in a RANKL/OPG molar ratio (16.2 ± 2.0) similar to that of the control (15.9 ± 0.8; Fig. 6*H*). We also performed a coculture assay using $OPG^{-/-}$ osteoblastic cells in order to analyze the effect of RANK-CRD-OPG-ΔCRD more precisely. Introduction of OPG-H significantly reduced the osteoclastogenic ability of $OPG^{-/-}$ osteoblastic cells, whereas introduction of RANK-CRD-OPG-ΔCRD reduced osteoclastogenic ability only slightly (Fig. 6*I*). However, recombinant protein added to the culture medium greatly inhibited the osteoclastogenic ability in both cases (Fig. 6*I*). These findings suggested that the selective inhibition of OPG function as a traffic regulator resulted in the upregulation of the osteoclastogenic ability of osteoblastic cells, meaning that OPG exerts its physiologic function as a traffic regulator for RANKL as well as a decoy receptor.

In the light of these results outlined, we have proposed a mechanism for RANKL subcellular trafficking in osteoblastic cells (Fig. 7*A*).

Discussion

Although it is widely accepted that RANKL plays a central role in osteoclastogenesis,[31–33] there are few reports focusing on RANKL in the context of subcellular protein trafficking in osteoblastic cells. Previously we have shown that the subcellular trafficking of RANKL in osteoblastic cells is strictly regulated via a series of complicated processes.[11] However, the mechanism that determines whether the newly synthesized RANKL is sorted to the pathway directed to the secretory lysosomes or that destined to the plasma membrane is still unclear. In this study we focused on OPG in the context of RANKL subcellular trafficking as a RANKL-interacting and osteoclastogenesis-regulating protein.-First, we analyzed the involvement of OPG in the subcellular trafficking of RANKL using nonosteoblastic HeLa cells (Fig. 1). A previous report has shown that the internalization of RANKL located at the plasma membrane is stimulated by addition of OPG to the culture medium in nonosteoblastic 293RL cells,[24] indicating the possibility that our observation that RANKL is localized in lysosomes when coexpressed with OPG in HeLa cells is due to internalization of RANKL from the cell surface. However, in the case of adding recombinant OPG-H protein to the culture medium simultaneously during GFP-RANKL expression, GFP-RANKL was still observed mainly on the plasma membrane (Fig. 1*D*), which is clearly different from that observed in the coexpression experiment (Fig. 1*B*). We also analyzed the necessity of each structural domain of OPG to exert its function as a traffic regulator for RANKL using various OPG mutants (Figs. 2 and 3). The results obtained indicated that the HBD of OPG is necessary for its function as a traffic regulator (Fig. 3*C*, *D*). Previous reports showed that anionic proteoglycans, such as heparan sulfate and keratan sulfate, can be detected in the Golgi apparatus in osteoblastic cells.[34,35] It also has been shown that OPG HBD can interact with heparin and heparan sulfate and that cationic HBD is necessary for interaction with anionic proteoglycans.[25,36] Considering these previous findings, interaction of OPG with anionic proteoglycans in the Golgi apparatus might be a necessary step for RANKL transport to lysosomes. As for the DDHDs, it is reported that the DDHDs contribute to the dimer stability of OPG based on a comparison of OPG-CRD (= only CRD) protein and OPG-ΔHBD (= CRD + DDHD) protein.[29] Considering this and the results of our coexpression experiments in HeLa cells using OPG-CRD (RANKL; plasma membrane), OPG-CRD-Fc (RANKL; plasma membrane), and OPG-ΔHBD (RANKL; Golgi apparatus), it appears that the DDHDs also contribute to the regulation of RANKL subcellular trafficking, which is not fully explained by the function of the DDHDs of stabilizing OPG dimer conformation. In addition, we also have shown that RANK-CRD-OPG-ΔCRD was not able to lead RANKL to lysosomes efficiently (Fig. 3*G*), indicating that the OPG CRD is indispensable for exerting OPG function as a traffic regulator for RANKL. Observations at high magnification showed that GFP-RANKL tended to exhibit a microclustered pattern at the plasma membrane in the case of addition of OPG-H to the medium (Fig. 1*D*), whereas GFP-RANKL exhibited rather a homogeneous

Fig. 6. Selective inhibition of endogenous OPG function as a traffic regulator for RANKL promoted the osteoclastogenic ability of osteoblastic cells. (*A*) GFP-RANKL and RANK-CRD-OPG-ΔCRD were expressed transiently in ST2 cells and coexpressed with LAMP1-KuOr (*upper panels*) or G58K-KuOr (*lower panels*). GFP-RANKL was observed mainly in the Golgi apparatus. (*B*, *C*) GFP-RANKL and RANK-CRD-OPG-ΔCRD were expressed transiently in $OPG^{+/+}$ (*B*) or $OPG^{-/-}$ (*C*) primary osteoblastic cells and coexpressed with LAMP1-KuOr (*upper panels*) or G58K-KuOr (*lower panels*). In both cases, GFP-RANKL was observed mainly in the Golgi apparatus. Each bar indicates 10 μm. (*D*) Cell surface expression level of GFP-RANKL in ST2 cells transiently coexpressing RANK-CRD-OPG-ΔCRD was quantitated by biotinylation assay. The cell lysates and cell surface proteins were subjected to immunoblotting and probed with anti-GFP antibody. Na^+,K^+-ATPase (a control membrane protein) was probed with an anti-Na^+,K^+-ATPase antibody. Compared with the control conditions, the cell surface amount of GFP-RANKL was increased markedly by coexpression of RANK-CRD-OPG-ΔCRD. (*E*) Coculture assay was performed using bone marrow cells and ST2 cells transiently expressing either OPG-H or RANK-CRD-OPG-ΔCRD. TRACP enzymatic activity, as an index of osteoclastogenic ability, was examined. The osteoclastogenic ability was increased significantly in the case of ST2 cells transfected with the RANK-CRD-OPG-ΔCRD construct, whereas the introduction of OPG-H slightly reduced the osteoclastogenic ability. Data are represented as mean ± SD. $^{*}p < .05$; $^{***}p < .001$. (*F*) Coculture assay was performed using bone marrow cells and ST2 cells. OPG-H or RANK-CRD-OPG-ΔCRD recombinant protein (100 ng/mL) was added to the culture medium. Under both conditions, TRACP enzymatic activity was markedly reduced. Data are represented as mean ± SD. $^{***}p < .001$. (*G*, *H*) Under these coculture assay conditions, the mRNA (*G*) and protein (*H*) expression levels of OPG and RANKL were measured. The protein level of OPG in the culture medium and cell lysates was determined by ELISA. Except for the overexpression of OPG-H, the mRNA and protein levels were almost the same. Data are represented as mean ± SD. (*I*) Coculture assay was performed using primary osteoblastic cells. Reduction of osteoclastogenic ability was greater in primary $OPG^{-/-}$ osteoblastic cells in which OPG-H was introduced than that observed in the case of RANK-CRD-OPG-ΔCRD introduction. However, OPG-H or RANK-CRD-OPG-ΔCRD recombinant protein (100 ng/mL) added in the culture medium showed a strong inhibitory effect on osteoclastogenesis. Data are represented as mean ± SD. $^{**}p < .01$; $^{***}p < .001$.

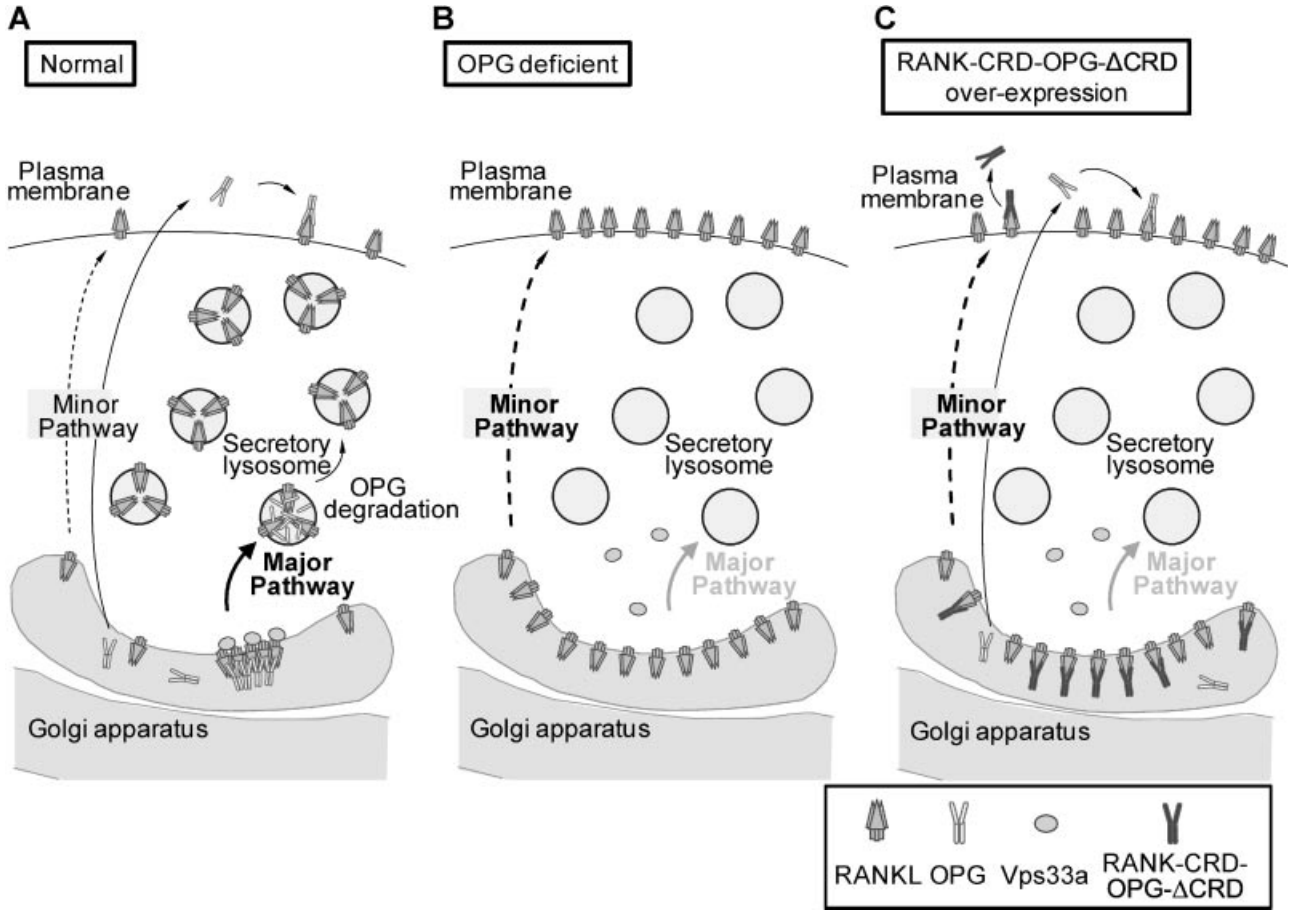

Fig. 7. Schematic diagram depicting the novel hypothesis of RANKL subcellular trafficking in osteoblastic cells. (*A*) Two pathways are considered to exist for RANKL expression on the plasma membrane in normal osteoblastic cells. The predominant pathway is from the Golgi apparatus to the secretory lysosomes, which requires the presence of both OPG and Vps33a. Stored RANKL is "decapped" via OPG degradation by lysosomal enzymes and is ready for translocation to the plasma membrane in response to stimulation. The other, minor pathway runs directly to the plasma membrane from the Golgi apparatus. (*B*) Under $OPG^{-/-}$ conditions, RANKL export via the major pathway, which requires OPG involvement in the Golgi apparatus, is unable to function. RANKL accumulation in the Golgi apparatus results in a marked increase in the amount of RANKL present on the cell surface. (*C*) RANK-CRD-OPG-ΔCRD selectively inhibits RANKL export through the major pathway involving endogenous OPG. RANKL accumulation in the Golgi apparatus results in an increase in the amount of RANKL present on the cell surface, as is the case in $OPG^{-/-}$ conditions.

distribution pattern at the plasma membrane when expressed alone in HeLa cells (Fig. 1*A*). This observation indicated that homodimeric OPG-H added to the medium cross-linked and gathered homotrimeric RANKL at the cell surface. The observation that the homogeneous distribution pattern of GFP-RANKL was not affected when monomeric OPG-CRD was added to the medium (data not shown) also supports the preceding explanation. Considering these findings, it is possible that dimeric OPG can cross-link and gather RANKL in the small area when copresent in the Golgi apparatus, which might be favorable for exporting RANKL from the Golgi apparatus efficiently. On the other hand, when recombinant RANK-CRD-OPG-ΔCRD protein was added to the medium, little clustering of RANKL at the plasma membrane was observed (data not shown), indicating that the property as a cross-linker for RANKL is much weaker in this chimeric protein. A previous report showing that the binding sites of RANKL to RANK and RANKL to OPG are somewhat different from each other[37] also supports the preceding observation. In addition, we also investigated the interaction between OPG and Vps33a because we have previously shown the involvement of Vps33a in the transfer of newly synthesized RANKL to the lysosomes in osteoblastic cells.[11] In our previous experiments, the interaction between RANKL and Vps33a was detected in ST2 cells, which express OPG endogenously.[11] In this study, immunoprecipitation analysis of HeLa cell lysates showed that the interaction between RANKL and Vps33a was greatly enhanced under OPG coexpression conditions (Fig. 5*D*). Considering all these facts, it is suggested that both the CRD and the HBDs are necessary for the efficient clustering of RANKL in the Golgi apparatus and that RANKL clustering contributes to the efficient recognition and export of RANKL by a pathway involving Vps33a. OPG as a cross-linker for RANKL might play an essential role in the determination of RANKL sorting.

Generally, most of the soluble acid hydrolases are modified with mannose 6-phosphate residues (M6P), allowing their recognition by M6P receptors (MPRs) in the Golgi apparatus and subsequent transport to the lysosomes.[38,39] Two MPR

isoforms, MPR46 [also called *cation-dependent MPR* (46 kDa)] and MPR300 [also called *cation-independent MPR* (300 kDa)], have been identified.[40] Based on these reports, we checked the involvement of MPRs in the RANKL trafficking pathway. However, treatment of HeLa cells with siRNA targeted to MPR46 and MPR300 did not affect RANKL transfer to the lysosomes under OPG coexpression conditions (data not shown). The RANKL and OPG mutant constructs lacking *N*-glycosylation also showed normal transfer to the lysosomes (data not shown), which also indicates that RANKL transfer to the lysosomes is independent of MPRs. Furthermore, we also examined the involvement of clathrin, which is known to be a part of various membrane trafficking processes, including the transfer of MPR-hydrolase complexes.[41] Treatment with siRNA targeting the clathrin heavy chain did not affect RANKL subcellular trafficking (data not shown). These results indicate that the RANKL export pathway from the Golgi apparatus to the lysosomes is independent of MPRs and clathrin.

Next, we investigated the traffic regulation of RANKL using mouse primary osteoblastic cells (Figs. 4 and 5). Except for the observation that RANKL is observed mainly in the Golgi apparatus in $OPG^{-/-}$ osteoblastic cells (Fig. 4*B*, *D*), the effect of OPG and its mutants on RANKL subcellular trafficking is almost consistent with the results obtained in the experiments using HeLa cells. Considering all the results in this and previous studies,[11] we can hypothesize that RANKL localized at the cell surface under nonstimulated conditions is involved in the signal input at the early stage of osteoclastogenesis, whereas RANKL stored in lysosomes is involved in the signal input at the later stage of osteoclastogenesis. As we mentioned earlier, the disruption of RANKL sorting in the Golgi apparatus resulted in a marked increase in RANKL at the cell surface and the loss of lysosomal RANKL storage in $OPG^{-/-}$ osteoblasts (Figs. 4 and 7*B*). In this situation, it is considered that the organized process of signal input is unable to function. Consequently, under *OPG* knockout conditions, the intensity of the RANKL signal is strong and constant throughout contact with osteoclast precursors, resulting in the excess activation of osteoclasts. On the other hand, RANKL behavior is strictly regulated in the presence of OPG coexpression (Fig. 4*A*). OPG localization observed in osteoblastic cells was almost identical with that of RANKL (Fig. 5*F*), indicating that OPG is transported to lysosomes in a complex with RANKL. In addition, quantitation by ELISA showed that the OPG detected in cell lysate is markedly increased by chloroquine treatment (Fig. 5*H*). We can hypothesize from these results that most of the newly synthesized RANKL is "capped" with OPG during protein synthesis, transported to lysosomes in "capped" form, and then "decapped" in lysosomes through OPG degradation by lysosomal enzymes. Decapped RANKL stored in lysosomes is ready for stimulating RANK after translocation to the cell surface.

As for the observation that RANKL is accumulated largely in the Golgi apparatus in $OPG^{-/-}$ osteoblastic cells (Fig. 4*B*, *D*), we could not reach a concrete conclusion in this report. RANKL introduced into nonosteoblastic cells showed plasma membrane expression, indicating the possibility that RANKL can be transported to the plasma membrane via the conventional secretion pathway. There is little information about the mechanism involved in the exporting steps from the Golgi apparatus and about the membrane protein delivered to the cell surface via the conventional pathway.[42] However, it is unlikely that transport of membrane protein to the cell surface is suppressed nonselectively in osteoblastic cells. Actually, Asgr1 introduced into osteoblastic cells exhibited plasma membrane expression with or without OPG expression (Fig. 5*A*). Therefore, it is considered that there are other unknown mechanisms involved in the suppression of RANKL export from the Golgi apparatus. This mechanism might act physiologically as a safety valve for the case that RANKL traffic regulation by OPG is unable to function in osteoblastic cells.

Finally, the physiologic significance of RANKL sorting in the Golgi apparatus should be discussed. The RANK-CRD-OPG-ΔCRD construct used in this study is a useful tool to evaluate OPG function because selective inhibition of endogenous OPG function as a traffic regulator for RANKL can be achieved by overexpression of this protein in osteoblastic cells (Fig. 6). Neither OPG expression at the mRNA level nor the total amount of endogenous OPG were affected by overexpression of this protein (Fig. 6*G*, *H*), indicating that the function of endogenous OPG as a decoy receptor for RANKL was not affected. The molar ratio of RANKL/OPG at the protein level was also unaffected (Fig. 6*H*). Moreover, we also showed that the recombinant protein of RANK-CRD-OPG-ΔCRD construct functions as an inhibitory decoy receptor for RANKL as well as OPG (Fig. 6*F*). Therefore, the number of molecules contributing to RANKL inhibition as a decoy receptor was considered to be greatly increased by the overexpression of RANK-CRD-OPG-ΔCRD (Fig. 7*C*). Despite the large increase in inhibitory decoy receptor molecules, the overexpression of RANK-CRD-OPG-ΔCRD in osteoblastic ST2 cells resulted in the significant upregulation of osteoclast formation in the coculture system with bone marrow cells (Fig. 6*E*). These results indicate that OPG function as a traffic regulator for RANKL is crucial at least as well as that as a decoy receptor.

In patients with juvenile Paget disease, several mutations in *OPG* have been reported. This disease presents in infancy or early childhood with pain from debilitating fractures and deformities owing to a markedly accelerated rate of bone remodeling throughout the skeleton.[43] Some missense mutations in the CRD are associated with severe osteoporotic phenotypes,[44,45] probably owing to the lack of ligand binding of OPG. In addition, a frameshift mutation (D198RfsX7) resulting in deletion of the DDHDs and the HBD has been reported in a severe juvenile Paget disease patient.[45] There is a possibility that mRNA stability was decreased and protein expression level of OPG also was decreased.[45] However, on the basis of this study, it can be explained that only the CRD form of OPG, which is synthesized according to the mutation, cannot regulate RANKL sorting in the Golgi apparatus, resulting in the marked increase in the amount of RANKL at the osteoblastic cell surface. Another frameshift mutation (D323FfsX4), resulting in deletion of the HBD, has been reported in a relatively mild juvenile Paget disease patient.[46] RANKL coexpressed with OPG-ΔHBD mutant tends to accumulate in the Golgi apparatus and is not transported to either lysosomes or the cell surface, as is shown in the experiments using HeLa cells. Considering this, the D323FfsX4 mutation might result in the retention of RANKL in the Golgi apparatus, and the

increase in the amount of RANKL transported to the cell surface is rather small compared with that in the case of the D198RfsX7 mutation.

In conclusion, we have found that OPG regulates RANKL sorting in the Golgi apparatus and that both the CRD and the HBD of OPG are necessary for this function. We also have shown that OPG function as a traffic regulator for RANKL is crucial for regulating osteoclastogenesis as well as that as a decoy receptor. Here we propose a scheme for RANKL subcellular trafficking in osteoblastic cells considering this and our previous report (Fig. 7*A*).

Disclosures

SA and MH contributed equally to this work. All the authors state that they have no conflicts of interest.

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